Succinate thiokinase from *Thermus aquaticus* and *Halobacterium salinarium*

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Both citrate synthase and succinate thiokinase occur in either a 'large' or 'small' form. The 'large' forms of these two enzymes have been found only in Gram-negative bacteria, whereas Gram-positive bacteria and eukaryotes contain the 'small' forms of the two. Hitherto, naturally-occurring organisms have only been found to contain either both 'large' or both 'small' forms. The bacteria *Thermus aquaticus* and *Halobacterium salinarium* produce 'small' citrate synthases. In the case of the Gram-negative organism *Thermus aquaticus*, this constitutes a clear exception to the general pattern. This work shows that both these bacteria contain 'large' succinate thiokinases, thus indicating that organisms do exist which contain mixed types of these two citric acid cycle enzymes.

Citrate synthase *Succinate thiokinase* Thermus aquaticus Halobacterium salinarium

1. INTRODUCTION

Our previous studies of the two acyl-CoA-utilizing enzymes of the citric acid cycle, citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) and succinate thiokinase (succinyl-CoA synthetase, EC 6.2.1.4 and 6.2.1.5), have revealed a striking correlation between their molecular structures and the taxonomic grouping of the source organism [1–5]. Both enzymes exist in Nature in either a 'large' or a 'small' form. Thus citrate synthases have M_r-values in the region of either 250 000 or 100 000, while the M_r-values of succinate thiokinases are around 150 000 or 75 000. In each case the 'large' enzyme is a feature exclusive to the Gram-negative bacteria; Gram-positive bacteria and eukaryotic organisms contain the 'small' enzyme.

In the case of citrate synthase, two exceptions to this general correlation have been reported. First it was found [6] that the citrate synthase of *Halobacterium cutirubrum* was of the 'small' type and this has also been found to be the case with other halobacteria [2]. These halophilic organisms were formerly grouped with the Gram-negative bacteria though they have recently been reclassified as members of the archaebacteria [7]. Secondly, it was reported [8] that *Thermus aquaticus* and other *Thermus*-like Gram-negative bacteria also produce only a 'small' citrate synthase. This anomalous behaviour may be related to the extreme growth conditions required by these two types of organisms. It therefore seemed worthwhile to examine the succinate thiokinases of these bacteria to see if their M_r-values parallel those of the citrate synthase in being of the 'small' type or whether these particular organisms are unusual in having 'small' citrate synthase but 'large' succinate thiokinase. The latter combination has been shown to be the case.

2. EXPERIMENTAL

T. aquaticus, strain YT-1 (ATCC 25104) was grown as in [8] and the harvested bacteria were stored as a frozen paste. To prepare an extract, 1.5 g of frozen cells were suspended in 4 ml of 0.1 M Na/K phosphate buffer (pH 7.0) and disrupted with an MSE 100 W sonicator operated for

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1.5 min at full power with cooling. Cell debris was removed by centrifuging at 25 000 × *g* for 10 min and the yellow supernatant solution was used without further purification. The protein content of this extract was 43 mg/ml and the specific activity of succinate thiokinase was 0.036 μmol.min^{-1}.mg protein^{-1}.

A culture of *H. salinarium* (NCMB 764) was kindly provided by Mr Peter N. Green (Torry Research Station, Aberdeen). Bacteria were grown at 37°C in the medium described in [9], harvested and stored as a frozen paste. To prepare an extract, 2.5 g of frozen cells were suspended in 2 ml of 50 mM Na/K phosphate buffer (pH 7.6) containing 4 M KCl and sonicated as above for 45 s. After centrifugation at 25 000 × *g* for 10 min the supernatant solution contained protein at a concentration of 100 mg/ml and succinate thiokinase at a spec. act. of 0.01 μmol.min^{-1}.mg protein^{-1}.

Succinate thiokinase was assayed polarographically as in [3]. For the enzyme from *T. aquaticus*, reaction mixtures contained 0.1 M Na/K phosphate (pH 8.0), 10 mM $MgCl_2$, 0.15 mM succinyl-CoA and 0.5 mM ADP. Similar mixtures were used for the enzyme from *H. salinarium*, but the buffer was 50 mM Na/K phosphate (pH 7.6) containing 4 M KCl. Citrate synthase was assayed spectrophotometrically at 412 nm as previously described [8], but 4 M KCl was included in the assay mixture in the case of the *H. salinarium* enzyme. Lactate dehydrogenase was assayed at 340 nm by following the rate of oxidation of NADH by pyruvate.

Gel filtration was performed at 4°C on a column (2.5 × 35 cm) of Sephadex G-200. In the case of *T. aquaticus* extract, the column was equilibrated and run with 0.1 M Na/K phosphate (pH 7.0) whereas 50 mM Na/K phosphate (pH 7.6) containing 1 M KCl was the buffer used with the *H. salinarium* preparation. Extract (2 ml), to which had been added 50 μl (0.25 mg) of lactate dehydrogenase (rabbit muscle; Boehringer), was applied to the column and fractions (35 drops, ~2 ml) were collected.

Thermal inactivation experiments were done by heating 0.2 ml portions of enzyme in pre-equilibrated glass tubes at various temperatures. After 5 min the tubes were cooled quickly in ice-water and the activity of succinate thiokinase was assayed as above.

3. RESULTS AND DISCUSSION

Succinate thiokinase activity was readily measurable in extracts of both *T. aquaticus* and *H. salinarium* and gel filtration with lactate dehydrogenase (M_r ~140 000) as marker protein provided an approximate measure of the M_r-values of these bacterial enzymes. Fig. 1 shows the pattern of elution of the *T. aquaticus* succinate thiokinase and citrate synthase. The position of elution of the succinate thiokinase shows it to be of the 'large' type and is consistent with a M_r-value in the region of 150 000. The citrate synthase, on the other hand, was eluted well after lactate dehydrogenase and is clearly a 'small' form of the enzyme, as previously reported [8].

In an earlier study of the citrate synthase from *T. aquaticus* it was shown that the enzyme was remarkably thermostable [8]. Examination of the thermostability of *T. aquaticus* succinate thiokinase has revealed a similar resistance to thermal inactivation (fig. 2) and indicates that the 'large' form of the enzyme poses no problem to the growth of this organism at high temperature.

An essentially identical pattern of elution to that shown in fig. 1 was obtained when the *H. salinarium* extract was run on the gel column,

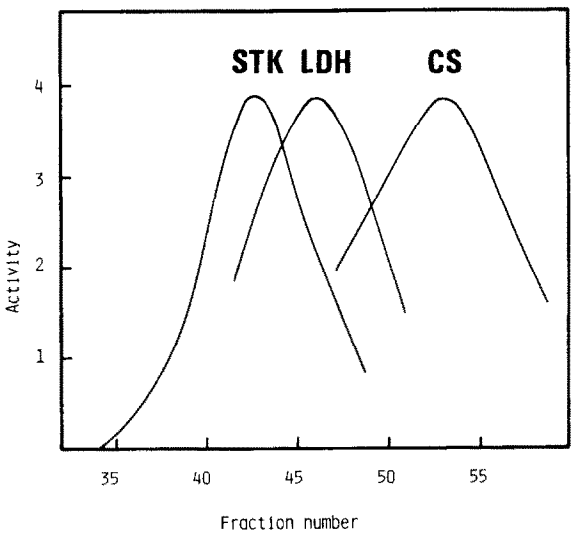

Fig. 1. Gel filtration of *T. aquaticus* enzymes. A cell-free extract was run on a column of Sephadex G-200 with lactate dehydrogenase (LDH) as in the text. STK, succinate thiokinase; CS, citrate synthase. Enzyme activities are in arbitrary units.

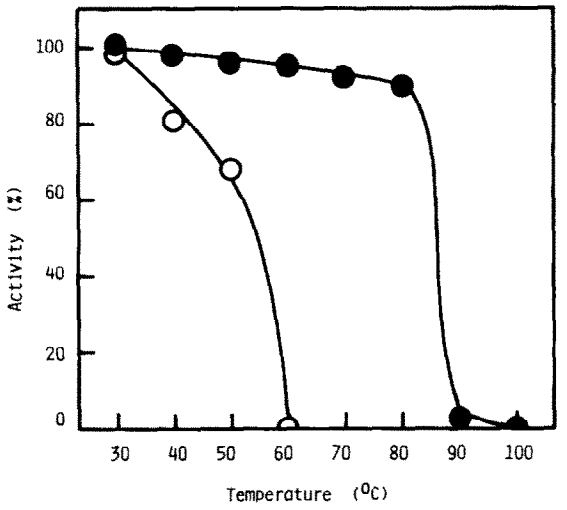

Fig. 2. Thermal inactivation of *T. aquaticus* succinate thiokinase. The enzyme was heated for 5 min at the temperatures indicated, cooled and assayed as described in the text. The behaviour of the enzyme from *Escherichia coli* after similar treatment is shown for comparison. (●) *T. aquaticus*; (○) *E. coli*.

showing that the halobacterial succinate thiokinase is also a 'large' type enzyme, and confirming the 'small' size of the citrate synthase.

It thus appears that both *T. aquaticus* and *H. salinarium* possess 'large' succinate thiokinases. In the case of *T. aquaticus* this result is consistent with the Gram-negative status of the organism, and in the case of the archaebacterium *H. salinarium* it is interesting that the enzyme resembles that of Gram-negative bacteria. The two bacteria studied here present exceptions to the general pattern derived from earlier studies [2,3] which indicated that diverse organisms contain either 'large' citrate synthase and 'large' succinate thiokinase or 'small' forms of both enzymes. Other than some artificially-mutated bacteria [10,11] the present results reveal that there are naturally-occurring bacteria which contain the Gram-positive form of citrate synthase but the Gram-negative form of succinate thiokinase, and it will be interesting to see how widespread this is.

The 'large' citrate synthases encountered in Gram-negative bacteria are generally sensitive to feedback inhibition by NADH (an end-product of the citric acid cycle); *T. aquaticus* and *H. salinarium*, by switching to the 'small' form of the enzyme, appear to have lost this particular metabolic control mechanism. All Gram-negative bacterial citrate synthases which are inhibited by NADH lose this sensitivity in the presence of salt [2]. This led to the speculation [6] that during the adaptation of halobacteria to halophilic life the NADH inhibition of citrate synthase became physiologically non-functional and, together with the particular subunit structure constituting the 'large' enzyme, was lost in the course of evolution. On the other hand, authors in [2] suggested that under the extreme conditions of growth required by *T. aquaticus* (optimum temperature ~70°C) and *Halobacterium* spp. (~4 M salt) a smaller enzyme molecule may be better able to maintain its native structure than a larger, more polymeric, one and that this might underlie the occurrence of the 'small', rather than the 'large', citrate synthase in these bacteria. However, the occurrence of 'large' succinate thiokinases in the extreme thermophile and halophile clearly limits the generality of this proposal but emphasizes the conserved nature of this molecular feature of the bacterial cell. It still remains to be established whether the 'large' succinate thiokinase confers any functional (regulatory?) advantage compared with the catalytically competent 'small' enzyme. Any such advantage might explain the preservation of the 'large' succinate thiokinases in these organisms.

ACKNOWLEDGEMENT

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